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ON THE MODE OF ACTION AND BIOCHEMICAL PROPERTIES OF ANTI-INFLAMMATORY DRUGS—I

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Abstract—The horse-radish peroxidase–hydrogen peroxide (HRP–H_2O_2) catalyzed oxidation of *o*-dianisidine was inhibited by indomethacin, oxyphenbutazone, mefenamic acid, phenylbutazone and *p*-acetamidophenol. The greatest inhibitory effect (ID_{50} 0·014 mM) was obtained with indomethacin, and its mode of action in this system has been examined further. The results obtained suggest that indomethacin acted by competing with *o*-dianisidine for peroxidase and hydrogen peroxide or peroxidase–hydrogen peroxide complexes.

MUCH recent interest on the mode of action of anti-inflammatory drugs has centred on the inhibition of prostaglandin synthesis from incubates of arachidonic acid with enzyme preparations from various animal tissues.[1–4] Other recent studies of u.v.- and hydrogen peroxide-induced lipid peroxidation and haemolysis in canine erythrocytes have suggested that anti-inflammatory drugs may absorb and destroy free radicals or peroxides.[5,6] To date, only a few reports[7] have appeared either on the role and nature of the enzyme systems and co-factors involved in lipid peroxidation and prostaglandin synthesis, or of their interactions with anti-inflammatory drugs both *in vivo* and *in vitro*. The present report is part of a study of these two phenomena, and describes effects of some potent non-steroidal anti-inflammatory drugs on the horse-radish peroxidase–hydrogen peroxide catalyzed oxidation of *o*-dianisidine.

MATERIALS AND METHODS

Materials. Horse-radish peroxidase (HRP, Rz 1·14) was obtained from Worthington Biochemical Corp.; hydrogen peroxide (H_2O_2, 30% w/v, Analar), *o*-dianisidine, acetylsalicylic acid and sodium salicylate from British Drug Houses; phenylbutazone and oxyphenbutazone from Geigy Pharmaceutical Co.; *p*-acetamido-phenol from Koch–Light & Co., and indomethacin from Merck, Sharp and Dohme. Mefenamic acid was kindly donated by Parke-Davis & Co., Ltd. Acetylsalicylic acid, indomethacin and mefenamic acid were used as their respective sodium salts. All other reagents used were of the highest grade commercially available.

Stock solutions. Enzyme: 500 μg/ml in 100 mM sodium phosphate buffer (pH 7·4). Immediately before using, 0·1 ml was diluted to 250 ml in phosphate buffer also containing 20 μg/ml of bovine serum albumin.

Dye. 0·25% *o*-dianisidine in methyl alcohol (freshly prepared in amber bottle).

Assay of peroxidase activity. Peroxidase activity was assayed at room temperature (21 ± 1°) with *o*-dianisidine as hydrogen donor using a system containing 0·041 mM

H_2O_2, 0·34 mM *o*-dianisidine (or 100 μl of 0·25% *o*-dianisidine), 0·04 μg of HRP (or 200 μl of the diluted enzyme solution), with and without a varying amount of the inhibitor, in a final volume of 3·0 ml of the phosphate buffer. The rate of *o*-dianisidine oxidation was followed spectrophotometrically at 460 nm by recording extinction (E) at 30 sec intervals against suitable controls. One unit of activity is that causing an increase in absorbancy of 0·001/min.

RESULTS

Indomethacin (0·014 mM), oxyphenbutazone (0·18 mM), mefanemic acid (0·80 mM), phenylbutazone (0·90 mM) and *p*-acetamidophenol (19·00 mM) all inhibited the peroxidase catalyzed oxidation of *o*-dianisidine in the presence of hydrogen–peroxide. The ID_{50}-values are given in parentheses. The results are shown in Fig. 1. Because indomethacin was the most potent of all the drugs tested its mode of action with HRP and H_2O_2 was investigated further. Acetylsalicylate and salicylate were inactive at the highest concentrations used (5 and 20 mM, respectively).

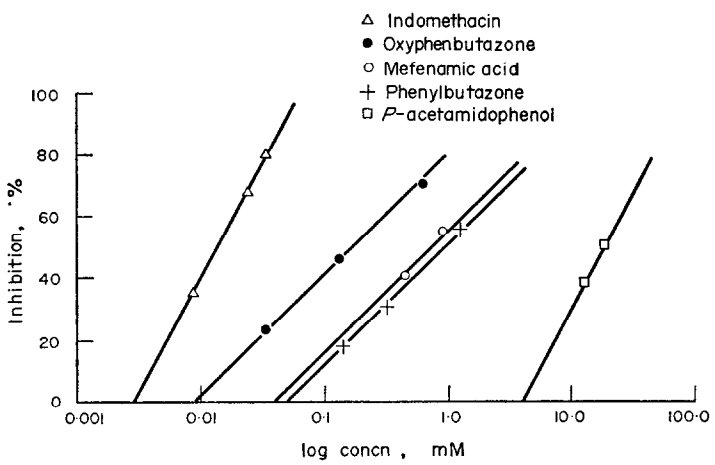

FIG. 1. Concentration (mM) of some non-steroidal anti-inflammatory drugs used (as shown), plotted on a log scale against the percentage inhibition of the oxidation of *o*-dianisidine by horse-radish peroxidase and H_2O_2.

Nature of inhibition. It was important to ascertain whether the reduced peroxidase activity in the presence of indomethacin was due to a non-specific denaturation of the protein or to a mass action combination between indomethacin and the enzyme. If a reversible peroxidase–indomethacin complex is formed, the equilibrium should be approachable from both directions. To test the reversibility of the indomethacin inhibition, an appropriate dilution (the same as used for the peroxidase assay) of the stock HRP solution (500 μg/ml) was made in 0·08 mM indomethacin (which produces complete inhibition); the peroxidase assay was then performed in the presence of less than 0·08 mM indomethacin. It was observed that the activity of the enzyme was restored after indomethacin was diluted out and that a given concentration of indomethacin elicits approximately the same inhibition whether added directly or whether added in a high concentration first and then diluted. It may be inferred that the inhibition of peroxidase by indomethacin is reversible in character.

Kinetics of the inhibition. The effect of indomethacin on the rate of reaction of the HRP–H_2O_2 system was determined with various concentrations of the substrate, *o*-dianisidine. The effect of indomethacin at various concentrations was then determined at each substrate concentration. The reciprocals of the substrate concentrations ($1/S$) and the reciprocals of the rates of utilization of H_2O_2 ($1/V$) are plotted in Fig. 2. The linear relationship between ($1/S$) and ($1/V$) demonstrated the applicability of the Lineweaver–Burk equation[8] to the reaction between *o*-dianisidine and HRP and H_2O_2. The effects of indomethacin demonstrate a competitive inhibition where the slope is increased but the intercept is unchanged. It is evident therefore that indomethacin acts as a competitive inhibitor of *o*-dianisidine oxidation by HRP and H_2O_2.

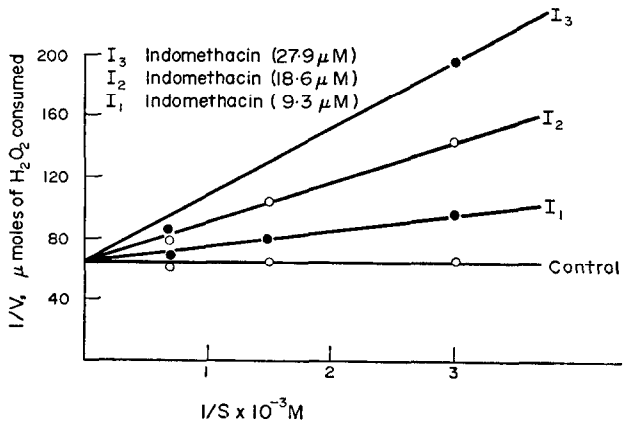

FIG. 2. Effect of indomethacin (I_1–I_3) on the rate of oxidation of *o*-dianisidine by horse-radish peroxidase and H_2O_2. The reciprocal substrate concentration is plotted against the reciprocal velocity.

Investigation of reducing properties. In the light of previous observations that indole-3-acetic acid can be oxidized by HRP and H_2O_2 or HRP and oxygen[9] and also that many sulfhydryl compounds are capable of reducing the coloured dyes formed by the actions of HRP and H_2O_2 on various substrates such as benzidine, guaiacol and *p*-phenylenediamine,[10] it was considered that the inhibition of *o*-dianisidine oxidation might be due to the reducing action of indomethacin on the dye formed by the reaction of HRP and *o*-dianisidine. To test the reducing power of indomethacin, it was added in 0·045 mM concentration at pH 7·4 to aliquots of oxidized *o*-dianisidine. The latter was made by reaction of H_2O_2 and HRP with *o*-dianisidine (see Methods) and removal of the excess H_2O_2 with catalase. However, indomethacin did not reduce the rust-red dye (oxidized *o*-dianisidine).

Effect on haem spectra. Haem enzymes show very characteristic absorption spectra, which are altered if some molecule such as an inhibitor combines with the haem portion of the enzyme.[11] An examination of the absorption spectra of HRP plus a molar excess of indomethacin showed no change, during 30 min, either qualitatively or quantitatively over the wavelength 260–700 nm. No evidence of a direct combination between indomethacin and HRP may therefore be obtained by spectrophotometric observation suggesting that indomethacin does not interact with the haem prosthetic group, but rather with the apoenzyme of HRP or with the HRP–H_2O_2 complex.

DISCUSSION

In both unsaturated lipid peroxidation and prostaglandin biosynthesis, the common steps have been postulated to involve consecutively, radical formation by hydrogen abstraction from an allylic position, uptake of oxygen to produce a peroxyradical and, finally, hydroperoxide formation. The findings reported above suggest that anti-inflammatory drugs may act at the stage of drug-peroxidase–hydroperoxide interaction. In the model system reported here, indomethacin, oxyphenbutazone, mefenamic acid, phenylbutazone and *p*-acetamidophenol are shown to inhibit the oxidation of *o*-dianisidine by HRP and H_2O_2 in approximately the same general order as their commonly known therapeutic efficacy.[12] Similarly guinea-pig lung microsomal peroxidase activity was also found to be inhibited by indomethacin. The concentrations required being of the same order as reported here (unpublished results).

Because neither aspirin (5 mM) nor sodium salicylate (20 mM) had any effect in the HRP–H_2O_2 system, these drugs may have a somewhat different mode of action from indomethacin; this is consistent with our observations with algesic substances.[13] Aspirin has also been shown to be ineffective in inhibiting H_2O_2-induced lipid peroxidation in canine erythrocytes.[5]

How anti-inflammatory drugs interfere with peroxidase–hydroperoxide interaction cannot be stated with certainty. Studies carried out with indomethacin (the most potent inhibitor of this system) suggest that these drugs may act by competing for HRP and H_2O_2 or highly reactive intermediate HRP–H_2O_2 compounds (antioxidant-like properties); but unlike certain antioxidants indomethacin itself did not show any reducing action on the coloured dye formed from *o*-dianisidine.

The above findings may have some direct pertinence to the mode of action of anti-inflammatory drugs in the inhibition of prostaglandin biosynthesis and lipid peroxidation for two reasons. First, competition between natural hydrogen donors, e.g. GSH (commonly involved in prostaglandin synthesis) and the drug for the available hydroperoxide or hydroperoxide–GSH peroxidase complexes may affect PG synthesis. Secondly, the drug may directly inactivate peroxides or lipid peroxides shown to be involved in pain[13,14] and experimental inflammation.[15] Further work along these lines using glutathione peroxidase–lipid hydroperoxide system will be necessary to obtain further knowledge of the underlying mechanism.

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